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PDE5 inhibitors, sildenafil and vardenafil, reverse multidrug resistance by inhibiting the efflux function of multidrug resistance protein 7 (ATP-binding Cassette C10) transporter

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Phosphodiesterase type 5 (PDE5) inhibitors are widely used in the treatment of male erectile dysfunction and pulmonary hypertension. Recently, several groups have evaluated the ability of PDE5 inhibitors for their anticancer activities. Previously, we had shown that sildenafil, vardenafil and tadalafil could reverse P-glycoprotein (ATP-binding cassette B1)-mediated MDR. In the present study, we determined whether these PDE5 inhibitors have the potential to reverse multidrug resistance protein 7 (MRP7; ATP-binding cassette C10)-mediated MDR. We found that sildenafil and vardenafil dose-dependently enhanced the sensitivity of *MRP7*-transfected HEK293 cells to paclitaxel, docetaxel and vinblastine, while tadalafil had only a minimal effect. Accumulation and efflux experiments demonstrated that sildenafil and vardenafil increased the intracellular accumulation of [^{3}H]-paclitaxel by inhibiting the efflux of [^{3}H]-paclitaxel in HEK/MRP7 cells. In addition, immunoblot and immunofluorescence analyses indicated that no significant alterations of MRP7 protein expression and localization in plasma membranes were found after treatment with sildenafil, vardenafil or tadalafil. These results demonstrate that sildenafil and vardenafil reverse MRP7-mediated a MDR through inhibition of the drug efflux function of MRP7. Our findings indicate a potentially novel use of PDE5 inhibitors as an adjuvant chemotherapeutic agent in clinical practice. (*Cancer Sci* 2012; 103: 1531–1537)

Multidrug resistance (MDR) occurs with mechanically and structurally unrelated drugs. MDR leads to the failure of cancer treatment by chemotherapeutic drugs, which are among the most effective treatment options for cancers.[1] One of the major mechanisms behind the simultaneous resistance is the efflux of different drugs mediated by ATP-binding cassette (ABC) transporters from cancer cells.[2] The ABC transporter superfamily are transmembrane proteins that are grouped into seven subfamilies (A–G) based on genome sequence similarities.[3] The ABCB1 (P-glycoprotein [P-gp]/MDR1), ABCG2 (BCRP/MXR) and ABCCs (multidrug resistance proteins [MRPs]) are the major players involved in mediating resistance to certain anticancer drugs. ABCB1 was the first discovered human ABC drug transporter, and transports a wide variety of hydrophobic compounds, including some of the most common anticancer drugs, such as taxanes, anthracyclines, vinca alkaloids and tyrosine kinase inhibitors (TKI).[4,5] The ABCG2 can also transport several anticancer drugs, such as antifolates, anthracyclines and TKI.[5–7] The nine MRP members (MRP1–MRP9) involved in MDR represent the major share of the 12 members of the C subfamily of the human ABC transporters.[8] The MRP subfamily can transport organic anions and anticancer drugs, such as anthracyclines, epipodophyllotoxins, vinca alkaloids and taxanes.[9] MRP7 (ABCC10), a member of the MRP subfamily, is similar in topology to MRP1, 2, 3 and 6, with two nucleotide-binding domains and three transmembrane domains.[10,11] MRP7 is able to confer resistance to several natural product chemotherapeutic drugs, including taxanes and vinca alkaloids, which are also substrates of P-gp.[12] MRP7 has been reported to confer resistance to vinorelbine and paclitaxel in non-small cell lung cancer cells[13,14] and to vincristine in human salivary gland adenocarcinoma cells.[15] Hopper-Borge *et al.* confirmed the *in vivo* functions of MRP7 using an *Mrp7* knockout mouse model. Their results suggested that Mrp7 could affect *in vivo* tissue sensitivity to taxanes.[16]

Inhibitors to ABC transporters might block transporter-mediated drug efflux function and re-sensitize MDR cancer cells to anticancer drugs.[17] Over the past three decades, numerous broad-spectrum or specific inhibitors of ABC transporters have been discovered and tested in *in vitro* and *in vivo* studies.[17] However, most of the ABC transporter inhibitors applied as chemosensitizers have not been used successfully in clinical cancer chemotherapy because of either adverse effects or toxic pharmacokinetic issues.[2] Another strategy for reversal agent development is discovering new functions of the drugs that are clinically approved. Recently, our group reported that several TKI and phosphodiesterase-5 (PDE5) inhibitors could reverse P-gp-mediated MDR.[18–23] Because MRP7 shares similar substrates and functions with P-gp, it is possible that P-gp modulators also overcome MRP7-mediated MDR. Indeed, most of the reported MRP7 inhibitors could inhibit the function of P-gp.[24–26] In the present study, we performed experiments using *MRP7*-transfected HEK293 cells to determine whether PDE5 inhibitors, such as sildenafil, vardenafil and tadalafil, could modulate MRP7-mediated MDR.

Materials and Methods

Materials. Sildenafil, vardenafil and tadalafil were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Cepharanthine was generously provided by Daiichi Sankyo Pharmaceutical (Tokyo, Japan). DMEM, FBS, penicillin/streptomycin and trypsin 0.25% were supplied by Hyclone (Logan, UT, USA). Monoclonal antibody 14C10 (against GAPDH) was acquired from Cell Signaling Technology (Danvers, MA,

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USA). Polyclonal antibody D-19 (against MRP7) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa flour 488 donkey anti-goat secondary antibody for immunocytochemistry was purchased from Molecular Probes (Eugene, OR, USA). [^{3}H]-paclitaxel (46.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). Paclitaxel, docetaxel, vinblastine, DMSO, MTT and other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

Cell lines and cell culture. The *MRP7* cDNA was generously provided by Dr Gary Kruh (University of Illinois, Chicago, IL, USA) and inserted into the pcDNA3.1 expression vector. The *MRP7* expression vector and parental plasmid were introduced into HEK293 cells by electroporation, as previously described.[11] Individual colonies were selected in medium containing G418 (1 mg/mL) and cultured for further analysis. All the cell lines were grown as adherent monolayers in flasks with DMEM supplemented with 10% bovine serum, 100 units/mL penicillin and 100 mg/mL streptomycin in a humidified incubator containing 5% CO_2 at 37°C.

Cytotoxicity assay. MTT colorimetric assay was performed to analyze the drug sensitivity, as previously described.[19] HEK293-pcDNA3.1 and HEK/MRP7 cells were seeded into 96-well plate in triplicate at 6000 cells/well and incubated in DMEM supplemented with 10% bovine serum at 37°C for 24 h. To determine the toxicity of PDE5 inhibitors, various concentrations of sildenafil, vardenafil and tadalafil diluted with medium were added into wells. To establish the reversal effect of PDE5 inhibitors on the sensitivity of anticancer drugs in MRP7-overexpressing cells, three different non-toxic concentrations of sildenafil, vardenafil and tadalafil (1.25, 2.5 and 5 μM) were added into plates 1 h prior to the addition of the substrates of MRP7 (paclitaxel, docetaxel and vinblastine). After drug incubation of 68 h, 20 μL MTT solution (4 mg/mL) was added into each well. The plates were further incubated for 4 h, then the medium was discarded, and 100 μL of DMSO was added into each well to dissolve the formazan crystals. The absorbance was determined at 570 nm by an OPSYS Microplate Reader from DYNEX Technologies (Chantilly, VA, USA). The degree of resistance was calculated by dividing the IC_{50} values (concentrations required to inhibit growth by 50%) for the HEK/MRP7 cells by those of the parental HEK293-pcDNA3.1 cells. The Bliss method was used to calculate the IC_{50} values according to survival curves.

[^{3}H]-paclitaxel accumulation and efflux. The effect of PDE5 inhibitors on the intracellular accumulation of paclitaxel in HEK293-pcDNA3.1 and HEK/MRP7 cell was measured using [^{3}H]-paclitaxel. HEK293-pcDNA3.1 and HEK/MRP7 cells were trypsinized and four aliquots from each cell line were suspended in the medium. Aliquots were pre-incubated with medium-only (control), sildenafil, vardenafil or tadalafil (5 μM each) at 37°C for 2 h, then incubated with 0.1 μM [^{3}H]-paclitaxel for another 2 h. For the efflux study, the cells were treated the same as in the drug accumulation study, and then washed three times with ice-cold PBS, suspended in fresh medium with or without PDE5 inhibitors. Aliquots were evenly collected at various time points (0, 30, 60 and 120 min). Samples from both accumulation and efflux experiments were washed by ice-cold PBS thrice and placed in scintillation fluid and radioactivity was measured in a Packard TRI-CARB 1900CA liquid scintillation analyzer from Packard Instrument Company (Downers Grove, IL, USA).

Preparation of total cell lysates and immunoblotting analysis. To determine the effect of PDE5 inhibitors on the expression of MRP7, HEK/MRP7 cells were incubated with 5 μM sildenafil, vardenafil or tadalafil for different time periods (0, 24, 48 and 72 h) then harvested and rinsed twice with cold PBS. The total cell lysates were collected with radioimmunoprecipitation assay buffer (Sigma Chemicals) (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin and 10 μg/mL leupeptin) for 30 min with occasional rocking followed by centrifugation at 13 000g at 4°C for 15 min. The protein concentration was determined by bicinchoninic acid-based protein assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of total cell lysates (40 μg of protein) were resolved by 4–12% SDS-PAGE and electrophoretically transferred onto PVDF membranes. After being incubated in blocking solution containing 5% skim milk in TBST buffer (10 mM Tris–HCL, PH 8.0, 150 mM NaCl and 0.1% Tween 20) at room temperature for 1 h, the membranes were immunoblotted overnight with primary antibodies anti-MRP7 (1:200 dilution) and anti-GAPDH (1:1000 dilution) at 4°C. Subsequently, the membranes were washed three times for 15 min with TBST buffer and incubated at room temperature for 2 h with HRP-conjugated secondary antibody (1:2000 dilution). The protein–antibody complex was detected using the enhanced Phototope TM-HRP Detection Kit (Cell Signaling Technology) and exposed to Kodak medical X-ray processor (Kodak, Rochester, NY, USA). The protein expression was quantified using Scion Image software (Scion, Frederick, MD, USA).

Immunofluorescence analysis. HEK/MRP7 cells (1×10^4) were seeded in 24-well plates and cultured overnight. Sildenafil, vardenafil or tadalafil at 5 μM were added into the wells and then cultured at 37°C for 72 h in a humidified incubator containing 5% CO_2. Cells were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and then rinsed with PBS three times. Non-specific reaction was blocked with 1% BSA for 1 h at room temperature. A polyclonal antibody D19 against MRP7 (1:200) was added and incubated overnight. Then, cells were incubated with Alexa Flour 488 donkey anti-goat IgG (1:2000) for 1 h at room temperature. DAPI was used for nuclear staining. Immunofluorescent images were taken using an inverted microscope (model IX70; Olympus, Center Valley, PA, USA) with IX-FLA fluorescence and a CCD camera.

Statistical analysis. All experiments were repeated at least three times and the differences were determined using Student's *t*-test. The statistical significance was determined at $P < 0.05$.

Results

Effects of PDE5 inhibitors on the sensitivity of anticancer drugs in the HEK293-pcDNA3.1 and HEK/MRP7 cells. Prior to analyzing the reversal efficacy of PDE5 inhibitors (sildenafil, vardenafil or tadalafil) on reversal MDR, we tested their cytotoxic effects in HEK293-pcDNA3.1 and HEK/MRP7 cell lines using the MTT assay. The results showed that the HEK/MRP7 cell lines did not confer significant resistance to three PDE5 inhibitors (Fig. S1). Then, we investigated the cytotoxicity of anti-cancer drugs (paclitaxel, docetaxel or vinblastine) alone and in combination with a PDE5 inhibitor (sildenafil, vardenafil or tadalafil; Fig. 1) at non-toxic concentrations (1.25, 2.5 and 5 μM) in the HEK293-pcDNA3.1 and HEK/MRP7 cells. As shown in Table 1 and Figure 2, HEK/MRP7 cells compared to parental HEK293-pcDNA3.1 cells exhibited a significant resistance to various MRP7 substrates, such as paclitaxel, docetaxel and vinblastine, which is consistent with our previous reports.[22] Sildenafil, vardenafil and tadalafil dose-dependently decreased the IC_{50} values of the abovementioned MRP7 substrates for HEK/MRP7 cells. However, tadalafil showed the smallest reversal effect. Cepharanthine, the known MRP7 inhibitor, as a positive control at 2.5 μM, completely reversed the resistance of HEK/MRP7 cells to paclitaxel, docetaxel and vinblastine. In contrast, sildenafil, vardenafil and tadalafil did not significantly reverse the resistance of HEK/MRP7 cells to cisplatin, a non-

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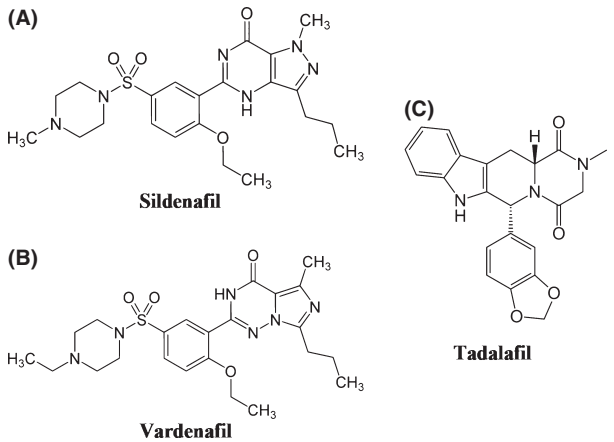

Fig. 1. Chemical structure of sildenafil (A), vardenafil (B) and tadalafil (C).

substrate of MRP7 ($P > 0.05$, Table 1, Fig. 2). In the parental HEK293-pcDNA3.1, the IC_{50} values of paclitaxel, docetaxel and vinblastine in the presence or absence of sildenafil, vardenafil or tadalafil showed no significant difference ($P > 0.05$; Table 1; Fig. 2).

PDE5 inhibitors increase the intracellular accumulation of [^{3}H]-paclitaxel in the HEK/MRP7 cells. To further confirm the effects of PDE5 inhibitors on the drug efflux function of MRP7, the intracellular accumulation of [^{3}H]-paclitaxel study was performed. The intracellular concentration of [^{3}H]-paclitaxel in HEK/MRP7 cells was significantly lower (28.5%) than that in parental HEK293-pcDNA3.1 cells (100%, Fig. 3). After the cells were incubated with either sildenafil, vardenafil or tadalafil at 5 μM for 2 h, the intracellular accumulation of [^{3}H]-paclitaxel in HEK/MRP7 cells was significantly increased by 3.3-, 3.7- and 2.1-fold, respectively, when compared to 2.5 μM of cepharanthine as a positive control by 3.8-fold (Fig. 3). Neither PDE5 inhibitors nor cepharanthine significantly affected the intracellular levels of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 cells (Fig. 3).

PDE5 inhibitors inhibit the efflux of [^{3}H]-paclitaxel mediated by MRP7 in HEK/MRP7. To ascertain whether the increase in the intracellular [^{3}H]-paclitaxel accumulation in the presence of sildenafil, vardenafil or tadalafil was due to the inhibition of [^{3}H]-paclitaxel efflux by MRP7, we designed a time course study to measure intracellular [^{3}H]-paclitaxel levels in the presence of sildenafil, vardenafil or tadalafil. As shown in Figure 4, HEK/MRP7 cells significantly extruded a higher percentage of intracellular [^{3}H]-paclitaxel than that in HEK293-pcDNA3.1 cells. However, in the presence of sildenafil, vardenafil or tadalafil at 5 μM, there was a significant decrease in the efflux of intracellular [^{3}H]-paclitaxel at different time periods (0, 30, 60 and 120 min) from HEK/MRP7 cells, but not from the parental HEK293-pcDNA3.1 cells. The intracellular accumulation of [^{3}H]-paclitaxel at 0 min was set as 100% and at 30, 60 and 120 min; the percentages were 62.43%, 39.47% and 23.44%, respectively, of the accumulated [^{3}H]-paclitaxel that remained in HEK/MRP7 cells in the absence of PDE5 inhibitors. When HEK/MRP7 cells were incubated with sildenafil, the percentage of the intracellular [^{3}H]-paclitaxel at 30, 60 and 120 min increased significantly to 90.62%, 82.14% and 62.35%, respectively (Fig. 4A). Vardenafil significantly increased the percentages of the intracellular [^{3}H]-paclitaxel at 30, 60 and 120 min to 95.34%, 93.58% and 63.92%, respectively (Fig. 4B). Meanwhile, at 30, 60 and 120 min, tadalafil significantly increased the percentage of [^{3}H]-paclitaxel accumulation to 76.44%, 63.41% and 46.48%, respectively (Fig. 4C). Sildenafil and vardenafil were more potent than tadalafil, which is consistent with the results in colorimetric growth assay and [^{3}H]-paclitaxel accumulation experiments.

Table 1. Effects of PDE5 inhibitors on the sensitivity of HEK293-pcDNA3.1 and HEK/multidrug resistance protein 7 (MRP7) cells to paclitaxel, docetaxel, vinblastine and cisplatin

Compounds	IC_{50} ± SD† (nM)	
	HEK 293-pcDNA-3.1	HEK/MRP7
Paclitaxel	11.64 ± 1.33 (1.00)‡	107.18 ± 11.25 (9.21)
+Sildenafil 1.25 μM	10.69 ± 1.04 (0.92)	45.47 ± 3.78 (3.91) **
+Sildenafil 2.5 μM	9.96 ± 0.92 (0.86)	28.35 ± 2.41 (2.44)**
+Sildenafil 5 μM	9.38 ± 0.86 (0.81)	13.37 ± 2.07 (1.15)**
+Vardenafil 1.25 μM	10.85 ± 1.27 (0.93)	34.85 ± 3.36 (2.99)**
+Vardenafil 2.5 μM	9.63 ± 0.89 (0.83)	19.86 ± 2.61 (1.71)**
+Vardenafil 5 μM	9.14 ± 1.01 (0.79)	12.39 ± 1.54 (1.06)**
+Tadalafil 1.25 μM	12.43 ± 0.95 (1.07)	93.78 ± 6.23 (8.06)
+Tadalafil 2.5 μM	11.41 ± 1.22 (0.98)	81.25 ± 7.16 (6.98)*
+Tadalafil 5 μM	10.84 ± 0.77 (0.93)	68.36 ± 5.83 (5.87)**
+Cepharanthine 2.5 μM	8.97 ± 1.18 (0.77)	11.81 ± 0.82 (1.01)**
Docetaxel	5.73 ± 0.65 (1.0)	64.81 ± 5.19 (11.31)
+Sildenafil 1.25 μM	5.35 ± 0.47 (0.93)	38.29 ± 3.75 (6.68)**
+Sildenafil 2.5 μM	5.28 ± 0.41 (0.92)	22.37 ± 2.97 (3.90)**
+Sildenafil 5 μM	4.74 ± 0.56 (0.83)	7.36 ± 0.83 (1.28)**
+Vardenafil 1.25 μM	5.72 ± 0.38 (1.0)	31.85 ± 3.62 (5.56)**
+Vardenafil 2.5 μM	4.88 ± 0.55 (0.85)	17.95 ± 2.57 (3.13)**
+Vardenafil 5 μM	4.59 ± 0.39 (0.80)	6.84 ± 0.79 (1.19)**
+Tadalafil 1.25 μM	6.26 ± 0.43 (1.09)	62.48 ± 5.03 (10.91)
+Tadalafil 2.5 μM	6.35 ± 0.59 (1.11)	55.21 ± 5.87 (9.64)
+Tadalafil 5 μM	5.86 ± 0.54 (1.02)	47.85 ± 3.98 (8.35)*
+Cepharanthine 2.5 μM	4.07 ± 0.52 (0.71)*	5.88 ± 0.41 (1.03)**
Vinblastine	11.19 ± 1.23 (1.0)	56.31 ± 4.61 (5.03)
+Sildenafil 1.25 μM	10.96 ± 0.95 (0.98)	36.19 ± 2.48 (3.23)**
+Sildenafil 2.5 μM	10.27 ± 0.82 (0.92)	25.92 ± 3.04 (2.32)**
+Sildenafil 5 μM	9.69 ± 0.97 (0.87)	14.37 ± 1.19 (1.28)**
+Vardenafil 1.25 μM	10.73 ± 1.11 (0.96)	33.72 ± 2.89 (3.01)**
+Vardenafil 2.5 μM	10.32 ± 1.03 (0.92)	21.45 ± 1.94 (1.92)**
+Vardenafil 5 μM	9.48 ± 0.98 (0.85)	12.39 ± 1.05 (1.11)**
+Tadalafil 1.25 μM	11.84 ± 0.88 (1.06)	51.28 ± 4.85 (4.58)
+Tadalafil 2.5 μM	11.51 ± 1.19 (1.03)	47.95 ± 4.11 (4.29)
+Tadalafil 5 μM	10.66 ± 1.08 (0.95)	40.54 ± 3.92 (3.62)*
+Cepharanthine 2.5 μM	8.92 ± 1.07 (0.80)	11.35 ± 0.93 (1.01)**
Cisplatin	1574.26 ± 84.95 (1.0)	1552.22 ± 74.39 (0.99)
+Sildenafil 5 μM	1730.35 ± 63.89 (1.10)	1629.48 ± 81.76 (1.04)
+Vardenafil 5 μM	1681.45 ± 102.34 (1.07)	1714.60 ± 71.43 (1.09)
+Tadalafil 5 μM	1746.87 ± 75.02 (1.11)	1757.12 ± 109.15 (1.12)
+Cepharanthine 2.5 μM	1677.38 ± 59.32 (1.07)	1635.30 ± 62.78 (1.04)

*$P < 0.05$; **$P < 0.01$. †IC_{50}: concentration that inhibited cell survival by 50%. Data are means ± SD of at least three independent experiments performed in triplicate. ‡Fold-resistance was determined by dividing the IC_{50} values of HEK/MRP7 cells by the IC_{50} values of HEK293-pcDNA3.1 cells in the absence or presence of sildenafil, vardenafil, tadalafil or cepharanthine.

PDE5 inhibitors do not alter the expression of MRP7. Reversal of MRP7-mediated MDR can be achieved by either altering MRP7 expression or inhibiting MRP7 function. To evaluate the effects of sildenafil, vardenafil or tadalafil on MRP7 expression, HEK/MRP7 cells were treated with sildenafil, vardenafil or tadalafil at 5 μM for 0, 24, 48 and 72 h, and the MRP7 expression levels were examined by western blot analysis. The results shown in Figure 5(A) indicate that none of the PDE5 inhibitors significantly alter the protein expression levels of MRP7 in HEK/MRP7 cells.

PDE5 inhibitors do not alter the localization of MRP7. Presumably, the transporters could be downregulated if they are

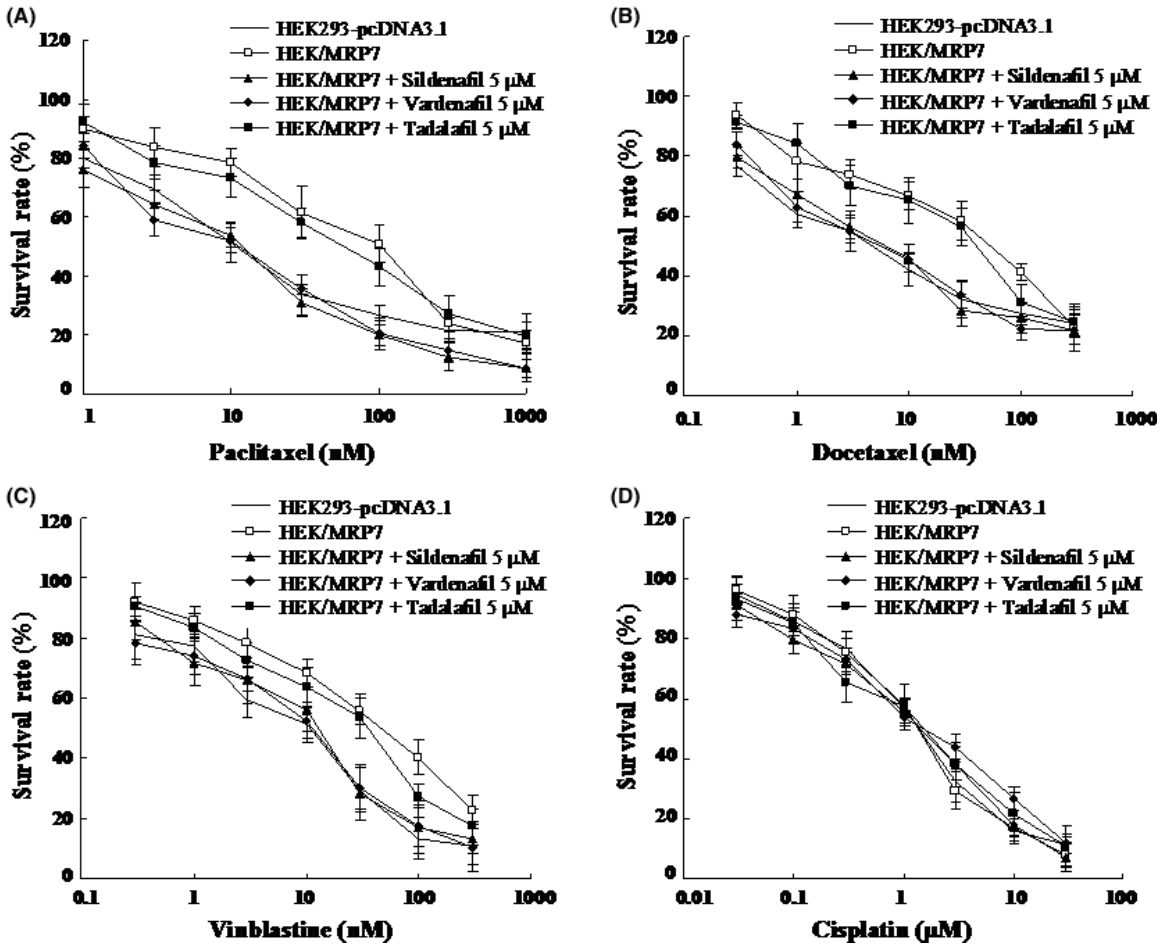

Fig. 2. Sildenafil and vardenafil reverse multidrug resistance protein 7 (MRP7)-mediated drug resistance in HEK/MRP7 cells. The survival curves of HEK/MRP7 cells in the presence or absence of sildenafil, vardenafil or tadalafilat at 5 μM and the parental HEK293-pcDNA3.1 cell at the different concentrations of (A) paclitaxel, (B) docetaxel, (C) vinblastine and (D) cisplatin, respectively. Cell survival was determined by MTT assay as described in the Materials and Methods. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times.

translocated or dislodged from plasma membrane to the cytosolic region. To rule out this possibility, we performed an immunofluorescence assay to examine whether the location of MRP7 was altered after the treatment with the PDE5 inhibitors. As shown in Figure 5(B), there was no alteration of MRP7 protein localization in plasma membranes after the treatment with sildenafil, vardenafil or tadalafil at 5 μM for 72 h. The western blotting (Fig. 5A) and immunocytochemical (Fig. 5B) experiments suggested that all three PDE5 inhibitors do not alter the expression and/or localization of the MRP7 transporter in HEK/MRP7 cells.

Discussion

Previously, we reported for the first time that three PDE5 inhibitors, sildenafil, vardenafil and tadalafil, could reverse P-gp-mediated MDR by directly inhibiting the transport function of P-gp.[22,23] Meanwhile, the efficacy of tadalafil as a reversal agent for P-gp was weaker than that of sildenafil and vardenafil. Furthermore, our *in vivo* experiments showed that sildenafil significantly enhanced the sensitivity of anticancer drugs on a P-gp-mediated MDR cancer xenograft model in nude mice (Amit K. Tiwari, Kamlesh Sodani, Chun-Ling Dai, Zhi-Jie Xiao, Zhe-Sheng Chen, unpublished data, 2012). In the present study, we examined whether sildenafil, vardenafil or tadalafil could reverse MRP7-mediated anticancer drug resistance. We chose well-established HEK293-pcDNA3.1 and HEK/MRP7 transfected cell lines.[25] The expression of MRP7 in HEK/MRP7 cell line was detected and confirmed by immunoblot analysis (data not shown).

The PDE5 inhibitors sildenafil and vardenafil were able to completely reverse the MDR mediated by MRP7, as evidenced with cytotoxicity assay data (Table 1). Sildenafil and vardenafil potently sensitized MRP7-overexpressing cells to MRP7 substrates paclitaxel, docetaxel and vinblastine. However, sildenafil and vardenafil did not sensitize the cells to cisplatin (non-substrate of MRP7) and had no significant effect on the drug sensitivity of the parental HEK293-pcDNA3.1 cells. Consistent with the cytotoxicity results, the drug accumulation data indicated that sildenafil and vardenafil significantly enhanced the intracellular accumulation of paclitaxel in HEK/MRP7 cells. Because MRP7 is a drug efflux pump that contributes to the decrease of intracellular paclitaxel concentrations, a time course efflux study was performed to further confirm the accumulation results. Indeed, the efflux study showed that the efflux of intracellular paclitaxel was significantly blocked by sildenafil and vardenafil in the HEK/MRP7 cell lines in comparison to those treated with no PDE5 inhibitors. Hence, the accumulation and efflux data along with cytotoxicity results indicate that sildenafil and vardenafil are targeting to MRP7 transporter.

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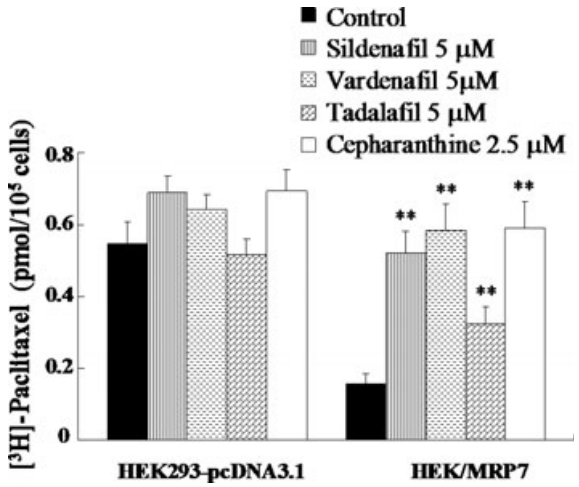

Fig. 3. Effects of sildenafil, vardenafil and tadalafil on the accumulation of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 and HEK/MRP7 cells. The intracellular accumulation of [^{3}H]-paclitaxel was measured by scintillation counting after cells were pre-incubated with or without sildenafil, vardenafil, tadalafil or cepharanthine for 2 h at 37°C and then incubated with 0.1 μM [^{3}H]-paclitaxel for another 2 h at 37°C. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times. $^{**}P < 0.01$, for values versus those in the control group.

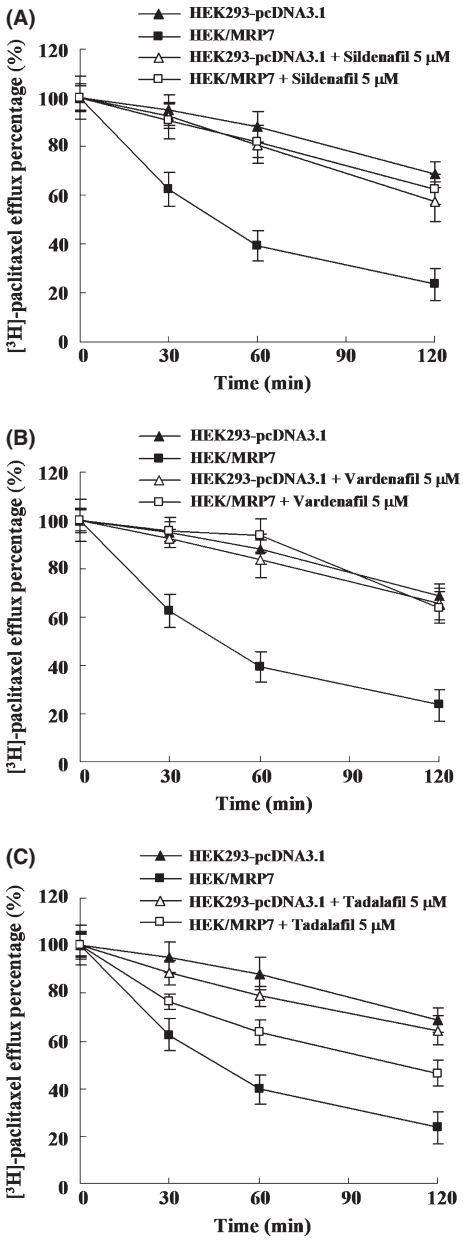

Fig. 4. Effects of sildenafil (A), vardenafil (B) and tadalafil (C) on the efflux of [^{3}H]-paclitaxel in HEK293-pcDNA3.1 and HEK/MRP7 cells. Cells were pre-incubated with or without sildenafil, vardenafil or tadalafil for 2 h at 37°C and further incubated with 0.1 μM [^{3}H]-paclitaxel for another 2 h at 37°C. Cells were then incubated in the fresh medium with or without the PDE5 inhibitors for different time periods at 37°C. After that, cells were collected and the intracellular levels of [^{3}H]-paclitaxel were measured by scintillation counting. A time course versus percentage of intracellular [^{3}H]-paclitaxel was plotted (0, 30, 60 and 120 min). Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times.

The reversal effect of MRP7-mediated MDR by sildenafil and vardenafil could be due to the inhibition of the MRP7 transporter function or the downregulation of the expression of the MRP7 transporter protein. The immunoblot and immunofluorescence analyses data rule out the second possibility, as no alterations in protein expression and localization of MRP7 transporter from plasma membranes were seen in HEK/MRP7 cells in the presence of sildenafil or vardenafil at 5 μM for up to 72 h. These findings further strengthen our results indicating that sildenafil and vardenafil inhibit MRP7 efflux function rather than downregulate MRP7 expression.

Sildenafil, vardenafil and tadalafil are cGMP-specific PDE5 competitive inhibitors that can prevent cGMP degradation. They are widely used in the treatment of male erectile dysfunction and pulmonary hypertension. These drugs have similar ring structure and are able to foster accumulation of the cellular cGMP, increasing the relaxation of vascular smooth muscle.[27] Several groups have evaluated the ability of PDE5 inhibitors in anticancer activities. PDE5 inhibitors are involved in antiproliferation and proapoptotic mechanism in multiple carcinomas.[28] Sarfati *et al.*[29] find that vardenafil and sildenafil can induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells *in vitro.* Moreover, Das *et al.*[30] report that sildenafil can enhance doxorubicin-induced apoptosis and upregulate caspase-3 and caspase-9 activities in prostate cancer cells. PDE5 inhibitors have also been reported to increase the tumor capillary permeability and to improve delivery of anticancer agents to brain tumors in a rat model.[31] In addition, PDE5 inhibitors can be used as modulators of the anticancer immune response and can reverse tumor-induced immunosuppression.[32] We have reported that sildenafil and vardenafil could enhance the anticancer drug sensitivity of cancer cells by reversing P-gp-mediated MDR.[22,23] These findings demonstrate a potentially novel use of PDE5 inhibitors as an adjuvant to chemotherapy and immune therapy.

However, another PDE5 inhibitor, tadalafil, can only significantly sensitize MRP7-overexpressing cells to chemotherapeutic drugs at 5 μM concentration, and its efficacy is weaker than that of sildenafil and vardenafil. Nonetheless, tadalafil has a longer half-life and duration of action than sildenafil and vardenafil in the clinic.[33] These differences might be due to their structural–activity relationship or their ability to inhibit other PDE enzymes. The molecular configuration of tadalafil departs entirely from that of both sildenafil and vardenafil (Fig. 1), whereas sildenafil and vardenafil differ only in particular by their nitrogen atoms in the heterocyclic ring system.[34–36] Furthermore, sildenafil and vardenafil inhibit PDE1 and PDE6 more significantly

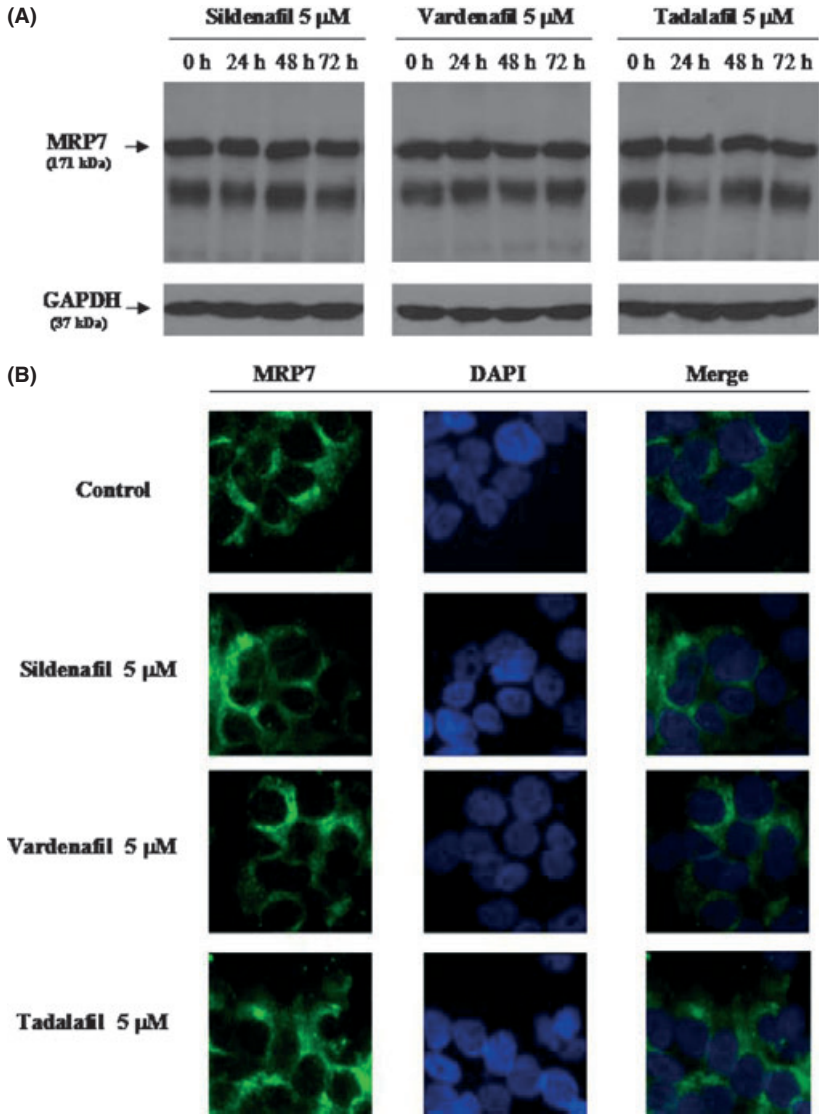

Fig. 5. Immunoblot detection (A) and immunofluorescence detection (B) of multidrug resistance protein 7 (MRP7) in HEK/MRP7 cells following incubation with PDE5 inhibitors. Cell lysates were prepared from HEK/MRP7 cells incubated with 5 μM sildenafil, vardenafil and tadalafil for different time periods (0, 24, 48, and 72 h). Immunoblot detection of MRP7 was done using polyclonal anti-MRP7 antibody and GAPDH was used as an internal control for equal loading. Equal amounts (40 μg of protein) of total cell lysates were used for each sample. The localization of MRP7 by immunofluorescence was done on paraformaldehyde fixed cells using polyclonal antibody D19 against MRP7 (1:200) and Alexa Flour 488 donkey anti-goat IgG (1:2000). Propidium iodide was used for nuclear counterstaining. Results from a representative experiment are shown. Similar results were obtained in two other trials.

than is the case for tadalafil.[37] Future studies are needed to investigate the interactions between PDE families and MRP7 and/or P-gp.

In conclusion, our findings demonstrate for the first time that sildenafil and vardenafil are able to reverse MRP7-mediated MDR by directly blocking the drug efflux function of MRP7 without altering MRP7 protein expression and localization from the plasma membranes. Hence, these PDE5 inhibitors might be inhibitors for multiple efflux pumps mediating MDR, such as P-gp and MRP7. In addition, sildenafil and vardenafil are already in clinical use, which makes them ideal candidates to be considered adjuvants to anticancer chemotherapy, especially in MRP7 and/or P-gp-mediated MDR.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Gottesman MM. Mechanisms of cancer drug resistance. *Annu Rev Med* 2002; **53**: 615–27.
2 Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* 2006; **5**: 219–34.
3 Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* 2002; **2**: 48–58.
4 Gottesman MM, Ambudkar SV. Overview: ABC transporters and human disease. *J Bioenerg Biomembr* 2001; **33**: 453–8.
5 Tiwari AK, Sodani K, Dai CL, Ashby CR Jr, Chen ZS. Revisiting the ABCs of multidrug resistance in cancer chemotherapy. *Curr Pharm Biotechnol* 2011; **12**: 570–94.
6 Doyle LA, Yang W, Abruzzo LV *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci USA* 1998; **95**: 15665–70.

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7 Maliepaard M, van Gastelen MA, de Jong LA *et al.* Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. *Cancer Res* 1999; **59**: 4559–63.
8 Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* 2001; **42**: 1007–17.
9 Chen ZS, Tiwari AK. Multidrug resistance proteins (MRPs/ABCCs) in cancer chemotherapy and genetic diseases. *FEBS J* 2011; **278**: 3226–45.
10 Kruh GD, Guo Y, Hopper-Borge E, Belinsky MG, Chen ZS. ABCC10, ABCC11, and ABCC12. *Pflugers Arch* 2007; **453**: 675–84.
11 Chen ZS, Hopper-Borge E, Belinsky MG, Shchaveleva I, Kotova E, Kruh GD. Characterization of the transport properties of human multidrug resistance protein 7 (MRP7, ABCC10). *Mol Pharmacol* 2003; **63**: 351–8.
12 Hopper-Borge E, Chen ZS, Shchaveleva I, Belinsky MG, Kruh GD. Analysis of the drug resistance profile of multidrug resistance protein 7 (ABCC10): resistance to docetaxel. *Cancer Res* 2004; **64**: 4927–30.
13 Bessho Y, Oguri T, Ozasa H *et al.* ABCC10/MRP7 is associated with vinorelbine resistance in non-small cell lung cancer. *Oncol Rep* 2009; **21**: 263–8.
14 Oguri T, Ozasa H, Uemura T *et al.* MRP7/ABCC10 expression is a predictive biomarker for the resistance to paclitaxel in non-small cell lung cancer. *Mol Cancer Ther* 2008; **7**: 1150–5.
15 Naramoto H, Uematsu T, Uchihashi T *et al.* Multidrug resistance-associated protein 7 expression is involved in cross-resistance to docetaxel in salivary gland adenocarcinoma cell lines. *Int J Oncol* 2007; **30**: 393–401.
16 Hopper-Borge EA, Churchill T, Paulose C *et al.* Contribution of Abcc10 (Mrp7) to in vivo paclitaxel resistance as assessed in Abcc10(–/–) mice. *Cancer Res* 2011; **71**: 3649–57.
17 Wu CP, Calcagno AM, Ambudkar SV. Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: evaluation of current strategies. *Curr Mol Pharmacol* 2008; **1**: 93–105.
18 Shi Z, Tiwari AK, Shukla S *et al.* Inhibiting the function of ABCB1 and ABCG2 by the EGFR tyrosine kinase inhibitor AG1478. *Biochem Pharmacol* 2009; **77**: 781–93.
19 Shi Z, Peng XX, Kim IW *et al.* Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance. *Cancer Res* 2007; **67**: 11012–20.
20 Dai CL, Tiwari AK, Wu CP *et al.* Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. *Cancer Res* 2008; **68**: 7905–14.
21 Tiwari AK, Sodani K, Wang SR *et al.* Nilotinib (AMN107, Tasigna) reverses multidrug resistance by inhibiting the activity of the ABCB1/Pgp and ABCG2/BCRP/MXR transporters. *Biochem Pharmacol* 2009; **78**: 153–61.
22 Shi Z, Tiwari AK, Shukla S *et al.* Sildenafil reverses ABCB1- and ABCG2-mediated chemotherapeutic drug resistance. *Cancer Res* 2011; **71**: 3029–41.
23 Ding PR, Tiwari AK, Ohnuma S *et al.* The phosphodiesterase-5 inhibitor vardenafil is a potent inhibitor of ABCB1/P-glycoprotein transporter. *PLoS ONE* 2011; **6**: e19329.
24 Zhou Y, Hopper-Borge E, Shen T *et al.* Cepharanthine is a potent reversal agent for MRP7(ABCC10)-mediated multidrug resistance. *Biochem Pharmacol* 2009; **77**: 993–1001.
25 Kuang YH, Shen T, Chen X *et al.* Lapatinib and erlotinib are potent reversal agents for MRP7 (ABCC10)-mediated multidrug resistance. *Biochem Pharmacol* 2010; **79**: 154–61.
26 Shen T, Kuang YH, Ashby CR *et al.* Imatinib and nilotinib reverse multidrug resistance in cancer cells by inhibiting the efflux activity of the MRP7 (ABCC10). *PLoS ONE* 2009; **4**: e7520.
27 Corbin JD, Francis SH. Molecular biology and pharmacology of PDE-5-inhibitor therapy for erectile dysfunction. *J Androl* 2003; **24**: S38–41.
28 Zhu B, Strada SJ. The novel functions of cGMP-specific phosphodiesterase 5 and its inhibitors in carcinoma cells and pulmonary/cardiovascular vessels. *Curr Top Med Chem* 2007; **7**: 437–54.
29 Sarfati M, Mateo V, Baudet S *et al.* Sildenafil and vardenafil, types 5 and 6 phosphodiesterase inhibitors, induce caspase-dependent apoptosis of B-chronic lymphocytic leukemia cells. *Blood* 2003; **101**: 265–9.
30 Das A, Durrant D, Mitchell C *et al.* Sildenafil increases chemotherapeutic efficacy of doxorubicin in prostate cancer and ameliorates cardiac dysfunction. *Proc Natl Acad Sci USA* 2010; **107**: 18202–7.
31 Black KL, Yin D, Ong JM *et al.* PDE5 inhibitors enhance tumor permeability and efficacy of chemotherapy in a rat brain tumor model. *Brain Res* 2008; **1230**: 290–302.
32 Serafini P, Meckel K, Kelso M *et al.* Phosphodiesterase-5 inhibition augments endogenous antitumor immunity by reducing myeloid-derived suppressor cell function. *J Exp Med* 2006; **203**: 2691–702.
33 Doggrell S. Do vardenafil and tadalafil have advantages over sildenafil in the treatment of erectile dysfunction? *Int J Impot Res* 2007; **19**: 281–95.
34 Turko IV, Ballard SA, Francis SH, Corbin JD. Inhibition of cyclic GMP-binding cyclic GMP-specific phosphodiesterase (Type 5) by sildenafil and related compounds. *Mol Pharmacol* 1999; **56**: 124–30.
35 Saenz de Tejada I, Angulo J, Cuevas P *et al.* The phosphodiesterase inhibitory selectivity and the in vitro and in vivo potency of the new PDE5 inhibitor vardenafil. *Int J Impot Res* 2001; **13**: 282–90.
36 Porst H. IC351 (tadalafil, Cialis): update on clinical experience. *Int J Impot Res* 2002; **14** (Suppl. 1): S57–64.
37 Bischoff E. Potency, selectivity, and consequences of nonselectivity of PDE inhibition. *Int J Impot Res* 2004; **16** (Suppl. 1): S11–4.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Survival curves of HEK293–pcDNA3.1 and HEK/multidrug resistance protein 7 (MRP7) at different concentrations of (A) sildenafil, (B) vardenafil and (C) tadalafil. Cell survival was determined by MTT assay, as described in the Materials and Methods. Data points represent the means ± SD of triplicate determinations. Experiments were performed at least three independent times.

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